PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 14/54, A61K 38/20, C12N 15/24, G01N 33/74 — A1

(11) International Publication Number: WO 96/18648

(43) International Publication Date: 20 June 1996 (20.06.96)

(21) International Application Number: PCT/IT95/00216

(22) International Filing Date: 13 December 1995 (13.12.95)

(30) Priority Data:
RM94A000805 14 December 1994 (14.12.94) IT

(71) Applicant *(for all designated States except US)*: ISTITUTO DI RICERCHE DI BIOLOGIA MOLECOLARE P. ANGELETTI S.P.A. [IT/IT]; Via Pontina Km. 30.600, I-00040 Pomezia (IT).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: CILIBERTO, Gennaro [IT/IT]; Viale Gorgia di Leontini, 330/19, I-00124 Casalpalocco (IT). SAVINO, Rocco [IT/IT]; Via della Tecnica, 76, I-00040 Roma (IT). LAHM, Armin [IT/IT]; Piazza S. Maria Ausiliatrice, 18, I-00153 Roma (IT). TONIATTI, Carlo [IT/IT]; Via Benedetto Croce, 26, I-00142 Roma (IT).

(74) Agents: DI CERBO, Mario et al.; Società Italiana Brevetti S.p.A., Piazza di Pietra, 39, I-00186 Roma (IT).

(81) Designated States: AU, CA, CN, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: SUPERAGONISTS AND ANTAGONISTS OF H IL-6, AND 3D MODELLING METHOD FOR THEIR SELECTION

(57) Abstract

It is known that the ligands of the group of cytokines similar to Interleukin 6 (IL-6), that is Oncostatin M (OSM), Leukemia Inhibitory Factor (LIF), Ciliary Neurothrophic Factor (CNTF) and Interleukin 11 (IL-11), induce the formation of a receptor complex of which the membrane molecule gp 130 is a part. The present invention refers to a methodology for selecting superagonists, antagonists and superantagonists of human interleukin-6 comprising the following operations: comparing the amino acid sequence of bovine granulocyte colony stimulating factor (bG-CSF) with the sequence of said hormone; and on the basis of the above comparison, formulating a three-dimensional model of said hormone, which allows the identification of residues that form the site of interaction with the specific receptor (Site 1) and those that constitute the site of interaction with gp 130 (Site 2) respectively. The invention allows the identification of these sites in human interleukin-6 and the isolation of variants having, with respect to the wild type hormone, a greater affinity for the specific receptor (superagonists and superantagonists) or affinity for gp 130 reduced or abolished (antagonists and superantagonists). The figure shows a scheme illustrating the methodoly applied to identify site 1 and site 2 in the case of human interleukin-6. The invention also describes the obtaining of specific superagonists and superantagonists of interleukin-6 and the use of superantagonists as low dose inhibitors of the growth of human myeloma cells dependent on wild type interleukin-6.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CS	Czechoslovakia
CZ	Czech Republic
DE	Germany
DK	Denmark
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgystan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakhstan
LI	Liechtenstein
LK	Sri Lanka
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SI	Slovenia
SK	Slovakia
SN	Senegal
TD	Chad
TG	Togo
TJ	Tajikistan
TT	Trinidad and Tobago
UA	Ukraine
US	United States of America
UZ	Uzbekistan
VN	Viet Nam

Superagonists and antagonists of h IL-6, and 3D modelling method for their selection

DESCRIPTION

The present invention relates to a methodology for selecting superagonists, antagonists and superantagonists of human interleukin-6 (hereinafter referred to also as h IL-6 or IL-6) based on three-dimensional modelling.

As is known, WO 92/21029 to Genentec Inc. teaches a method for determination of agonists or antagonists of growth hormones and ligands with a similar structural conformation. The potential agonists and antagonists are put into contact with a receptor for the hormone and this causes formation of a ternary complex consisting of a molecule of the potential agonist or antagonist and two molecules of such receptor for the hormone to be agonized or antagonized. Dimerization of receptors induced by a ligand molecule allows to conclude that the ligand has two different interaction sites (site 1 and site 2), on which it is possible to operate using mutagenesis to generate agonists or antagonists.

It is known that the ligands in the group of cytokines similar to Interleukin 6 (IL-6), that is Oncostatin M (OSM), Leukemia Inhibitory Factor (LIF), Ciliary Neurotrophic Factor (CNTF), and Interleukin 11 (IL-11), induce the formation of a receptor complex of which the membrane molecule gp 130 is a part. In this receptor complex the specific receptor for each of these cytokines and the membrane molecule gp 130 are always present as common elements. It is thus possible to formulate the hypothesis that site 1 and site 2 bind to two different molecules in this class of hormones: site 1 to the specific receptor and site 2 to gp 130. Identification of the two sites is made possible, as will be seen more clearly from the following, by construction of a three-dimensional model of the receptor complex based on the functional similarity between sequences of

the human growth hormone (hGH) receptor and sequences of the receptors for the hormones in question. Isolation of variants that, with respect to the wild type hormone, have a greater affinity for the specific receptor (superagonists or superantagonists) is obtained by construction of filamentous phage libraries, for example M13, carrying the hormone, both in the wild type and mutant version.

According to the invention, the difference between the three-dimensional model, for example of IL-6, adopted here and the one adopted in WO92/21029 leads to identify different residues in helix A and C as constituents of site 2. In fact, according to present invention, for the construction of the IL-6 model not the growth hormone, but the structure of a different cytokine was used as template.

Modelling of the human interleukin 6 molecule is performed as follows. It is known, from data available in scientific literature, that the amino acidic sequence of human interleukin 6 shows similarities with that of the granulocyte colony stimulating factor (G-CSF). The three-dimensional structure of bovine granulocyte colony stimulating factor (bG-CSF), determined using X-ray crystallography, was used as template to develop a three-dimensional model of human IL-6 from residue 16 to 184. Firstly, the amino acidic sequence of human IL-6 was aligned with that of bG-CSF. On the basis of the derived alignment, the amino acidic residues in the bG-CSF three-dimensional structure were replaced by the corresponding residues of human IL-6 using molecular modelling program in a computerized interactive graphic unit. In the positions in which alignment involves either deletions or insertions (which suggests a different local structure in the interleukin 6 molecule) adjustments were made by applying the options provided by the molecular modelling program.

This three-dimensional model of interleukin 6, based on the bG-CSF structure, has enabled the identification of the two sites of interaction between human interleukin 6 and its two receptors: the low affinity receptor gp 80 (site 1) and the high affinity signal transducer receptor gp 130 (site 2). The following procedure was used to identify the two sites. From sequence comparison it is known that all the members of the family of hematopoietic receptors are related to each other by the fact that they share a domain, known as the cytokine binding domain. This similarity of sequences also indicates a high probability of structural similarity in corresponding parts of the various receptors, including the two interleukin 6 receptors, gp 80 and gp 130. The observation that the cytokines that bind to these receptors all have (or are predicted to have) a similar structure, that is a four helix bundle, strongly supports the notion that the interaction between these cytokines and their receptors, by means of the cytokine binding domain, must be very similar - albeit not identical - in biologically active complexes.

Considering that the three-dimensional structure of one of these receptor complexes (the complex made by growth hormone and the extra-cellular domain of the dimeric receptor for the growth hormone, i.e. GHbp) has been determined by means of X-ray crystallography, our bG-CSF built model of human interleukin 6 allows us to identify the potential sites of interaction between interleukin 6 and its two receptors gp 80 (site 1) and gp 130 (site 2). This has been accomplished, according to the present invention, by constructing a structural model of gp 80 and gp 130 based on the coordinates furnished by the X-ray christallographic structure of the growth hormone receptor, and by substituting in such complex the growth hormone with our bG-CSF built model of human interleukin-6 (see fig. 1).

As is known, interleukin 6 is a polypeptide of 184 amino acids which, as described, belongs to the class of helical cytokines. Interleukin 6 is a multi-functional cytokine produced by various cell types. It acts as a differentiation and growth factor on cells of various lineages, such as for example cells in the immune system, hepatocytes, kidney cells, hematopoietic stem cells, keratinocytes and neurones.

Production of superagonists of interleukin 6 would allow the use of therapeutic doses lower than those required with wild type interleukin 6 in the treatment of numerous serious diseases. In fact, interleukin 6 has important and promising applications in the treatment of breast cancer, leukemia, and infectious diseases or diseases connected with disorders of bone marrow progenitor cells.

In addition superagonists of IL-6 could be used in protocols for ex vivo expansion of hematopoietic progenitor cells both in bone marrow transplantation and gene therapy.

On the other hand the production of antagonists or superantagonists of human interleukin 6 would allow inhibition of interleukin 6 in numerous diseases characterized by its excessive production, such as chronic autoimmune diseases, myeloma/plasmacytoma, post-menopausal osteoporosis and cancer cachexia.

The methodology for the selection of superagonists, antagonists or superantagonists of interleukin-6, according to the present invention, comprises the following operations:

- comparing the amino acid sequence of bovine granulocyte colony stimulating factor (bG-CSF) with the sequence of said hormone; and
- on the basis of the above comparison, formulating a three dimensional model of said hormone, which allows the identification of residues that form the site of interaction with the specific receptor (Site 1)

and those that constitute the site of interaction with gp 130 (Site 2) respectively.

For selection of superagonists of interleukin 6, the methodology according to the present invention further comprises the following additional operations:

- production of a series of phage libraries containing mutations of the following wild type residues of interleukin 6 (present in the form of fusion product with filamentous phage proteins):

helix A:
Ser 22, Glu 23, Asp 26, Arg 30, Leu 33, Ser 37, Arg 40, Glu 42;
loop AB:
Ser 52, Ser 53, Ala 56, Leu 57, Glu 59, Asn 60, Leu 62, Leu 64, Pro 65, Lys 66,
Met 67, Ala 68, Glu 69, Lys 70, Asp 71, Phe 74, Gln 75, Ser 76;
helix D:
His 164, Leu 165, Arg 168, Ser 169, Lys 171, Glu 172, Phe 173, Gln 175, Ser 176, Ser 177, Leu 178, Arg 179, Ala 180, Leu 181, Arg 182, Gln 183, Met 184.

- selection, from the mixed population of phages belonging to each individual phage library and expressing interleukin 6 mutants, of that or those with an affinity for the specific receptor greater than that of wild type interleukin; and

- identification of the best receptor binder amino acid sequence or sequences by sequencing of the DNA extracted from the selected phage particles.

In this case, a series of phage libraries can be produced containing mutations of said wild type residues of interleukin 6 present as a fusion product with the M13 pIII protein.

The methodology for selecting antagonists of interleukin 6 according to the present invention comprises - along with the operations indicated above for

molecular modelling of the human IL-6 protein and its receptor chains - the following operations:

- mutagenesis of the residues identified to form part of the site of interaction with gp 130 (Arg 30, Tyr 31, Gly 35, Ser 37, Ala 38, Ser 118, Lys 120, Val 121, Gln 124, Phe 125, Gln 127, Lys 128 and Lys 129), using conventional molecular biology techniques;

- evaluation of biological activity and affinity for the specific interleukin 6 receptor of the mutants produced as above, in order to identify variants of interleukin 6 whose affinity for the specific receptor is normal and that show reduction or loss of the biological activity; and

- evaluation of the above variants of interleukin 6 as antagonists for the biological activity of wild type interleukin 6 on human cell lines.

In case of obtaining of superantagonists of interleukin 6 by combination of the variants of amino acid sequences responsible for antagonist activity, identified as above, with amino acid mutations responsible for an increased affinity of the specific receptor for interleukin 6.

In the methodology for obtaining antagonists or superantagonists of interleukin 6, the mutagenesis of the residues identified as above can be performed using a molecular biology technique chosen from the group comprising Polymerase Chain Reaction, Primer Extension, Oligonucleotide Directed Mutagenesis, and combinations thereof.

The present invention is not limited to the methodology for selection of superagonists, antagonists or superantagonists of interleukin 6. On the contrary, it extends to molecules obtainable by said methodology of selection, i.e. to: superagonists of h IL-6, with the exception of the molecule called IL-6 IRA and carrying the following three substitutions Gln175Ile/Ser176Arg/Gln183Ala; antagonists of h IL-6,

with the exception of three molecules with the following substitutions:
Tyr31Asp/Gly35Phe/Ser118Arg/Val121Asp (DFRD)
Tyr31Asp/Gly35Phe/Ser118Phe/Val121Asp (DFFD)
Tyr31Asp/Gly35Phe/Ser118Leu/Val121Asp (DFLD);
and superantagonists of h IL-6, with the exception of the molecule called Sant1 and carrying the following seven substitutions: Tyr31Asp/Gly35Phe/Ser118Arg/Val121Asp/Gln175Ile/Ser176Arg/Gln183Ala.

Up to this point a general description of the subject of the present invention has been given. With the aid of the following examples a detailed description of specific embodiments of the invention will now be given, with the purpose of giving a better understanding of the objects, characteristics, advantages and methods of application thereof.

Figure 1 shows a scheme illustrating the methodology applied to identify site 1 and site 2 in the case of human interleukin 6.

Figure 2 shows the increase in potency of three superantagonists according to the invention, i.e. Sant 3, Sant 4 and Sant 5, over antagonist Tyr31Asp/Gly35Phe/Ser118Arg/Val121Asp (the one letter codes have been used in the figure), with the increase of concentration.

DEPOSITS

E.Coli K12 bacteria - transformed using the plasmid pHenΔhIL-6 containing, from the recognition site of the restriction enzyme SalI to that for the restriction enzyme NotI, a nucleotidic sequence coding for the amino acid sequence of wild type human interleukin 6 - have been deposited on 10/6/1993 with The National Collection of Industrial and Marine Bacteria Ltd. (NCIMB), Aberdeen, Scotland, UK, with access number NCIMB 40563.

Example 1

Application of the methodology according to the present invention for the selection of superagonists os

interleukin 6 by means of mutagenesis of amino acid residues in the AB loop

The strategy consists in construction of a hybrid gene containing all the region coding for hIL-6 (SEQ ID NO:1) followed by the last 157 amino acids of protein pIII of the phage M13 and preceded by the sequence Pel B, which vectors the synthesized protein to the periplasmic space.

This construct allows the obtaining of phagemid particles displaying on their surface correctly folded and biologically active human interleukin 6.

A phage library was constructed containing mutations of residues Asp 71, Phe 74, Gln 75 and Ser 76 of interleukin 6, starting from the variant IL-6 IRA (substitutions Gln175Ile/Ser176Arg/Gln183Ala) described in WO95/00852 and having an affinity for the receptor approximately five times greater that that of wild type human interleukin 6, present in the form of fusion product with protein pIII of filamentous phage M13. The library was constructed using the Primer Extension technique. The mutagenic oligonucleotide is IL-6 DFQS, a 95 nucleotides oligo, whose sequence is SEQ ID NO: 2. Primer IL-6 DFQS introduces degenerations into codons coding for the amino acids 71 (wild type Asp), 74 (wild type Phe), 75 (wild type Gln) and 76 (wild type Ser). The oligonucleotide IL-6 AB primer, whose sequence is SEQ ID NO: 3, was used as primer for the Primer Extension reaction. The two oligonucleotides were annealed in vitro, and the annealed oligonucleotides were used as substrate for a Primer Extension reaction. The double-stranded DNA fragment thus obtained was then digested and ligated into the plasmid pHenΔhIL-6 in order to replace the wild type sequence with the mutated ones. The ligation product was inserted in bacteria, yielding roughly three million independent transformants. The transformed bacteria were infected with the M13K07 helper

bacteriophage to generate the phage library (a library of phasmids).

The library underwent selection by incubation with magnetic beads coated with monoclonal antibody directed against shrIL-6R and in the presence of shrIL-6R and shrgp130. The phasmid population eluted at pH 3.6 was then amplified in bacteria. After four cycles of selection-amplification, randomly selected phasmids were sequenced over the mutagenized region, the corresponding mutant interleukin 6 proteins were produced in the periplasmic space of the appropriate bacterial strain and tested for interleukin 6 specific receptor binding. Table 1 shows that, using the methodology according to the present invention, it is possible to select variants of interleukin 6 having an additional increase in the affinity for the specific receptor, molecules with mutations both in helix D and in region A-B.

TABLE 1

Receptor binding properties in variants of interleukin 6-IRA containing additional mutations in the residues 71, 74, 75 and 76 of the region A-B

Position	71	74	75	76	Receptor binding (%)
wild type	Asp	Phe	Gln	Ser	100%
IL-6IRA	Asp	Phe	Gln	Ser	450%
phasmid D3-3	Asp	Tyr	Phe	Ile	2350%
phasmid D4-1	Asp	Tyr	Tyr	Val	2750%
phasmid D3-7	Asp	Phe	Tyr	Ile	2770%
phasmid D4-19	Asp	Phe	Tyr	Ser	1800%
phasmid D4-20	Asp	Phe	Tyr	Lys	4200%
phasmid D3-16	Asp	Phe	Tyr	Leu	1450%
phasmid D4-17	Asp	Phe	Phe	Ile	2430%

Example 2

Application of the methodology according to the present invention for obtaining superantagonists of interleukin 6

The four mutations Tyr31Asp/Gly35Phe/Ser118Arg/Val121Asp (DFRD) confer antagonistic properties as described in WO95/00852. These four mutations were combined with mutations capable of increasing the specific receptor binding capacity (described in example 1), using the Polymerase Chain Reaction (PCR) molecular biology technique. More specifically:

the super-binder mutations on helix D and region AB of the phasmid D 3-7 (described in example 1), to create the mutant protein Sant 3;

the super-binder mutations on helix D and region AB of the phasmid D 3-3 (described in example 1), to create the mutant protein Sant 4;

the super-binder mutations on helix D and region AB of the phasmid D 4-20 (described in example 1), to create the mutant protein Sant 5.

The mutant proteins, containing nine (Sant 3 and Sant 5) or ten (Sant 4) amino acid substitutions, were tested both for their specific interleukin-6 receptor binding, and for their ability to antagonize the biological activity of interleukin-6 on human hepatoma and myeloma cells. Table 2 and fig. 2 show the specific receptor binding properties of DFRD and of Sant 3, Sant 4 and Sant 5 along with the concentrations (expressed in nanograms of mutant per milliliter of culture medium) of mutant necessary to inhibit 50% of interleukin 6 biological activity (hepatoma cells were stimulated with 4 nanograms of wild type interleukin 6 per milliliter of culture medium, while myeloma cells were stimulated with 0.1 nanograms of interleukin 6 per milliliter of culture medium, due to the higher sensitivity of the latter cells to wild type interleukin 6).

TABLE 2

Inhibition of wild type interleukin 6 biological activity on both human hepatoma and myeloma cells as a function of the mutant antagonists' specific interleukin-6 receptor binding capacity

Antagonist	Receptor binding (% of wild type)	50% inhibition of interleukin 6 activity on: hepatoma cells Hep3B	myeloma cells XG-1
DFRD	97%	164 ng/ml	190.0 ng/ml
Sant 3	2800%	2.4 ng/ml	1.85 ng/ml
Sant 4	2000%	2.7 ng/ml	3.90 ng/ml
Sant 5	4500%	2.3 ng/ml	2.45 ng/ml

As can be seen from the table, the introduction of the amino acid substitutions described in example 1 has at once increased the specific receptor binding capacity of the parental mutant DFRD and decreased the amount of antagonist needed to inhibit 50% of wild type interleukin 6 biological activity on both cell lines tested, therefore generating very effective and strong interleukin 6 superantagonists.

SEQUENCE LISTING

GENERAL INFORMATION

(i) APPLICANT: ISTITUTO DI RICERCHE DI BIOLOGIA MOLECOLARE P. ANGELETTI S.p.A.

(ii) TITLE OF INVENTION: A METHODOLOGY FOR SELECTING SUPERAGONISTS, ANTAGONISTS AND SUPERANTAGONISTS OF HUMAN INTERLEUKIN-6 BASED ON RECEPTOR COMPLEX THREE DIMENSIONAL MODELLING

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Società Italiana Brevetti

(B) STREET: Piazza di Pietra, 39

(C) CITY: Rome

(D) COUNTRY: Italy

(E) POSTAL CODE: I-00186

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk 3.5" 1.44 MBYTES

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC DOS/MS DOS Rev 6.22

(D) SOFTWARE: Microsoft Word 6.0

(viii) ATTORNEY INFORMATION

(A) NAME: DI CERBO, Mario (Dr.)

(B) REGISTRATION NUMBER:

(C) REFERENCE: RM/X88471/PC-DC

(ix) TELECOMMUNICATION INFORMATION

(A) TELEPHONE: 06/6785941

(B) TELEFAX: 06/6794692

(C) TELEX: 612287 ROPAT

(1) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 555 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: no

(iv) ANTISENSE: no

(v) FRAGMENT TYPE: internal

(vii) IMMEDIATE SOURCE:

(A) SYNTHESIS: production in bacteria

(ix) FEATURE:

(A) NAME: IL-6 cDNA

(C) IDENTIFICATION METHOD: polyacrylamide gel

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
CCA GTA CCC CCA GGA GAA GAT TCC AAA GAT GTA GCC GCC CCA CAC AGA  48
Pro Val Pro Pro Gly Glu Asp Ser Lys Asp Val Ala Ala Pro His Arg
1               5                  10                  15
CAG CCS CTC ACG AGC TCA GAA CGA ATT GAC AAA CAA ATT CGG TAC ATC  96
Gln Pro Leu Thr Ser Ser Glu Arg Ile Asp Lys Gln Ile Arg Tyr Ile
            20                  25                  30
CTC GAC GGC ATC TCA GCC TTA AGA AAG GAG ACA TGT AAC AAG AGT AAC 144
Leu Asp Gly Ile Ser Ala Leu Arg Lys Glu Thr Cys Asn Lys Ser Asn
        35                  40                  45
ATG TGT GAA AGC AGC AAA GAG GCA CTG GCA GAA AAC AAC CTG AAC CTT 192
Met Cys Glu Ser Ser Lys Glu Ala Leu Ala Glu Asn Asn Leu Asn Leu
    50                  55                  60
CCA AAG ATG GCT GAA AAA GAT GGA TGC TTC CAA TCT GGA TTC AAT GAG 240
Pro Lys Met Ala Glu Lys Asp Gly Cys Phe Gln Ser Gly Phe Asn Glu
65                  70                  75                  80
GAG ACT TGC CTG GTG AAA ATC ATC ACT GGT CTT TTG GAG TTT GAG GTA 288
Glu Thr Cys Leu Val Lys Ile Ile Thr Gly Leu Leu Glu Phe Glu Val
                85                  90                  95
TAC CTA GAG TAC CTC CAG AAC AGA TTT GAG AGT AGT GAG GAA CAA GCC 336
Tyr Leu Glu Tyr Leu Gln Asn Arg Phe Glu Ser Ser Glu Glu Gln Ala
            100                 105                 110
AGA GCT GTC CAG ATG AGT ACA AAA GTC CTG ATC CAG TTC CTG CAG AAA 384
Arg Ala Val Gln Met Ser Thr Lys Val Leu Ile Gln Phe Leu Gln Lys
        115                 120                 125
AAG GCA AAG AAT CTA GAT GCA ATA ACC ACC CCT GAC CCA ACC ACA AAT 432
Lys Ala Lys Asn Leu Asp Ala Ile Thr Thr Pro Asp Pro Thr Thr Asn
    130                 135                 140
```

GCC AGC CTG CTG ACG AAG CTG CAG GCA CAG AAC CAG TGG CTG CAG GAC 480
Ala Ser Leu Leu Thr Lys Leu Gln Ala Gln Asn Gln Trp Leu Gln Asp
145 150 155 160
ATG ACA ACT CAT CTC ATT CTG AGA TCT TTT AAG GAG TTC CTG CAG TCC 528
Met Thr Thr His Leu Ile Leu Arg Ser Phe Lys Glu Phe Leu Gln Ser
165 170 175
AGC CTG AGG GCT CTT CGG CAA ATG TAG 555
Ser Leu Arg Ala Leu Arg Gln Met
180

(2) INFORMATION FOR SEQ ID NO: 2

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 95 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(iii) HYPOTHETICAL: no

(iv) ANTISENSE: no

(v) FRAGMENT TYPE: internal

(vii) IMMEDIATE SOURCE:
(A) SYNTHESIS: oligonucleotide synthesizer

(ix) FEATURE:
(A) NAME: DFQS
(C) IDENTIFICATION METHOD: polyacrylamide gel

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:
GTGAGAGCTC CAAAGAGGCA CTGGCAGAAA ACAACCTGAA CCTTCCAAAG ATGGCTGAAA 60
AANNSGGATG CNNSNNSNNS GGATTCAATG AGGAG 95

(3) INFORMATION FOR SEQ ID NO: 3

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(iii) HYPOTHETICAL: no

(iv) ANTISENSE: yes

(v) FRAGMENT TYPE: internal

(vii) IMMEDIATE SOURCE:

(A) SYNTHESIS: oligonucleotide synthesizer

(ix) FEATURE:

(A) NAME: AB primer

(C) IDENTIFICATION METHOD: polyacrylamide gel

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GGCCTCTAGA TATACCTCAA ACTCCAAAAG ACCAGTGATG ATTTTCACCA GGCAAGTCTC 60

CTCATTGAAT CC 72

CLAIMS

1. A methodology for selecting superagonists, antagonists and superantagonists of interleukin 6 comprising the following operations:

- comparing the amino acid sequences of bovine granulocyte colony stimulating factor (bG-CSF) with the sequence of said hormone; and

- on the basis of the above comparison, formulating a three-dimensional model of said hormone, which allows the identification of residues that form the site of interaction with the specific receptor (site 1) and those that constitute the site of interaction with gp 130 (site 2) respectively.

2. The methodology for selecting superagonists of interleukin 6 according to claim 1, further comprising the following additional operations:

- production of a series of phage libraries containing mutations of the following wild type residues of interleukin 6 present in the form of a fusion product with filamentous phage proteins Ser 22, Glu 23, Asp 26, Arg 30, Leu 33, Ser 37, Arg 40, Glu 42, Ser 52, Ser 53, Ala 56, Leu 57, Glu 59, Asn 60, Leu 62, Leu 64, Pro 65, Lys 66, Met 67, Ala 68, Glu 69, Lys 70, Asp 71, Phe 74, Gln 75, Ser 76, His 164, Leu 165, Arg 168, Ser 169, Lys 171, Glu 172, Phe 173, Gln 175, Ser 176, Ser 177, Leu 178, Arg 179, Ala 180, Leu 181, Arg 182, Gln 183, Met 184;

- selection, from the mixed population of phages expressing interleukin 6 mutants, of that or those with an affinity for the specific receptor greater than that of wild type interleukin; and

- identification of the best amino acid sequence or sequences binding the receptor by sequencing of the DNA extracted from the selected phage particles,

with the exception of the h IL-6 molecule carrying the three substitutions Gln175Ile/Ser176Arg/Gln183Ala.

3. The methodology for selecting superagonists of interleukin 6 according to claim 2, in which a series of phage libraries are produced, containing mutants of said wild type residue of interleukin 6 present as a product of fusion with protein pIII of M13.

4. The methodology for selecting antagonists of interleukin 6 according to claim 1, further comprising the following additional operations:

- mutation of the residues identified in claim 1, to form part of the site of interaction with gp 130 (Arg 30, Tyr 31, Gly 35, Ser 37, Ala 38, Ser 118, Lys 120, Val 121, Gln 124, Phe 125, Gln 127, Lys 128 and Lys 129), using conventional molecular biology techniques;

- evaluation of biological activity and affinity with the specific interleukin 6 receptor of the mutants produced as above, in order to identify variants of interleukin 6 whose affinity to the specific receptor is intact and that show reduction or loss of the biological activity; and

- evaluation of the above variants of interleukin 6 as antagonists for the biological activity of wild type interleukin 6,

with the exception of the three h IL-6 molecules with the following substitutions:

Tyr31Asp/Gly35Phe/Ser118Arg/Val121Asp
Tyr31Asp/Gly35Phe/Ser118Phe/Val121Asp
Tyr31Asp/Gly35Phe/Ser118Leu/Val121Asp.

5. The methodology for selecting superantagonists of interleukin 6 according to claims 2 to 4 by combination of the variations of amino acid sequences responsible for antagonist activity, indicated above, with amino acid variations responsible for an increased affinity of the specific receptor for interleukin 6, with the exception of the h IL-6 molecule carrying the seven substitutions Tyr31Asp/Gly35Phe/Ser118Arg/Val121Asp/Gln175Ile/Ser176Arg /Gln183Ala

6. The methodology for selecting antagonists or superantagonists of interleukin 6 according to claim 5, in which the mutagenesis of the residues identified as above is performed using a molecular biology technique chosen from the group comprising Polymerase Chain Reaction, Primer Extension, Oligonucleotide Directed Mutagenesis, and combinations thereof.

7. Interleukin 6 mutants according to claim 3, both showing an increased affinity for the specific receptor and containing the mutations Glutamine 175 Isoleucine, Serine 176 Arginine and Glutamine 183 Alanine, together with multiple substitutions in the Phenylalanine 74, Glutamine 75 and Serine 76 residues.

8. Interleukin-6 mutants according to claim 7, showing an increased affinity for the specific receptor and containing mutations chosen from the group comprising:

- Glutamine 75 Tyrosine, Serine 76 Isoleucine, Glutamine 175 Isoleucine, Serine 176 Arginine and Glutamine 183 Alanine;
- Phenylalanine 74 Tyrosine, Glutamine 75 Phenylalanine, Serine 76 Isoleucine, Glutamine 175 Isoleucine, Serine 176 Arginine and Glutamine 183 Alanine; and
- Glutamine 75 Tyrosine, Serine 76 Lysine, Glutamine 175 Isoleucine, Serine 176 Arginine and Glutamine 183 Alanine, said mutants exhibiting an affinity for the specific receptor increased 27.7 times, 23.5 times and 42 times, respectively.

9. Human interleukin-6 mutants having simultaneous substitution of the residues 31, 35, 74, 75, 76, 118, 121, 175, 176, 183 obtainable from claims 4 to 6 and which, by combining their antagonist properties to a greater affinity for the receptor, have the effect of superantagonists at low doses.

10. Human interleukin-6 mutants according to claim 9, with mutations chosen from the group comprising:

- Tyrosine 31 Aspartic acid, Glycine 35 Phenylalanine, Serine 118 Arginine, Valine 121 Aspartic acid, Glutamine 75 Tyrosine, Serine 76 Isoleucine, Glutamine 175 Isoleucine, Serine 176 Arginine and Glutamine 183 Alanine;

- Tyrosine 31 Aspartic acid, Glycine 35 Phenylalanine, Serine 118 Arginine, Valine 121 Aspartic acid, Phenylalanine 74 Tyrosine, Glutamine 75 Phenylalanine, Serine 76 Isoleucine, Glutamine 175 Isoleucine, Serine 176 Arginine and Glutamine 183 Alanine; and

- Tyrosine 31 Aspartic acid, Glycine 35 Phenylalanine, Serine 118 Arginine, Valine 121 Aspartic acid, Glutamine 75 Tyrosine, Serine 76 Lysine, Glutamine 175 Isoleucine, Serine 176 Arginine and Glutamine 183 Alanine,

said mutants being capable of inhibiting the biological activity of wild type interleukin 6 on sensitive human cells including myeloma cells the growth of which is IL-6-dependent.

11. Use of the superagonists according to claim 7 or 8 for the preparation of drugs for therapy of trombocytopenia in man and for the ex vivo expansion of human hematopoietic progenitor cells for bone marrow transplantation and gene therapy.

12. Use of the interleukin-6 mutants according to claim 10 for the preparation of drugs for treatment of diseases characterized by overproduction of interleukin-6, and in particular of multiple myeloma, reumatoid arthritis, postmenopausal osteoporosis and systemic lupus erythematosus.

Construction of the Model

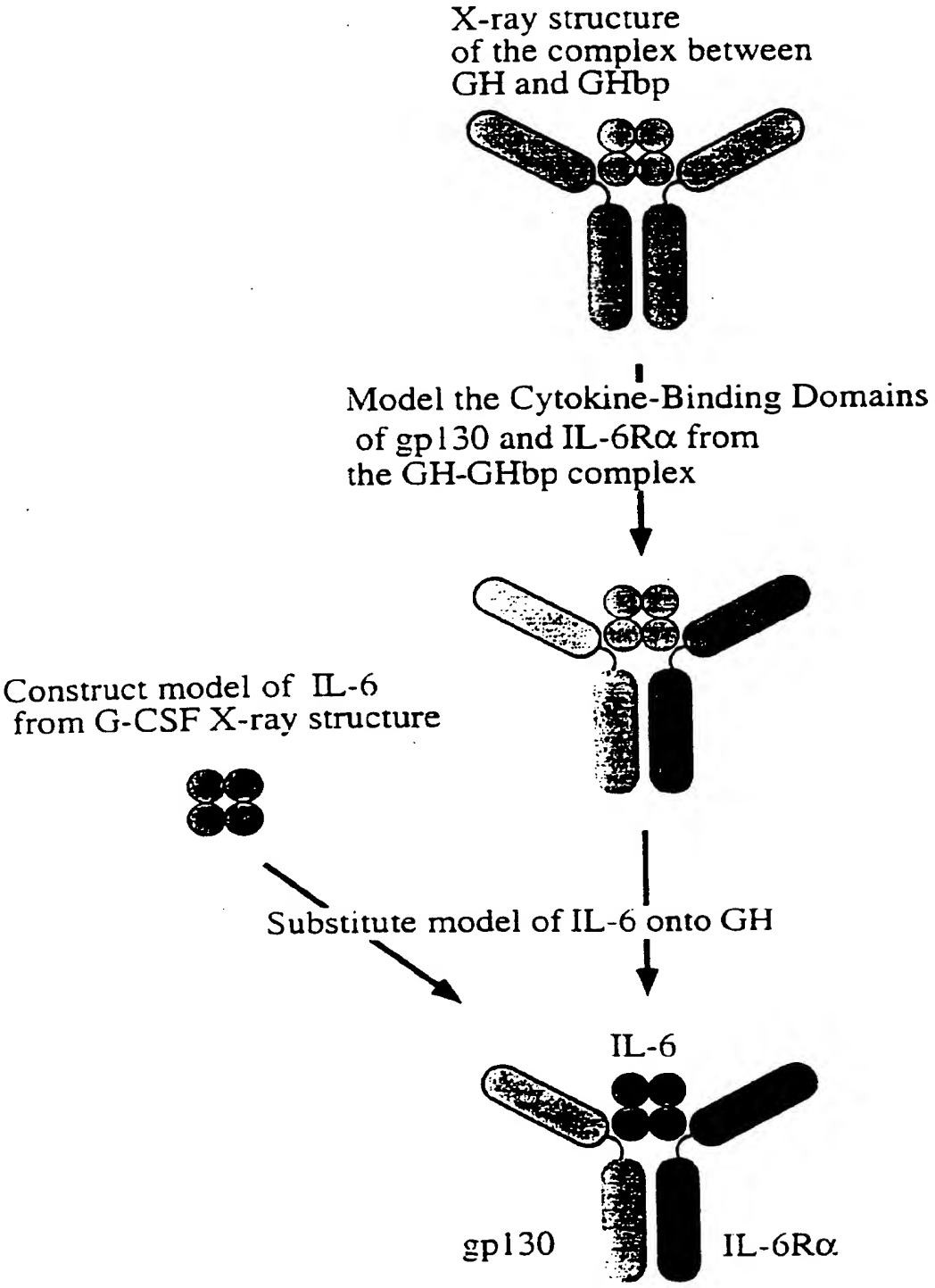

FIG. 1

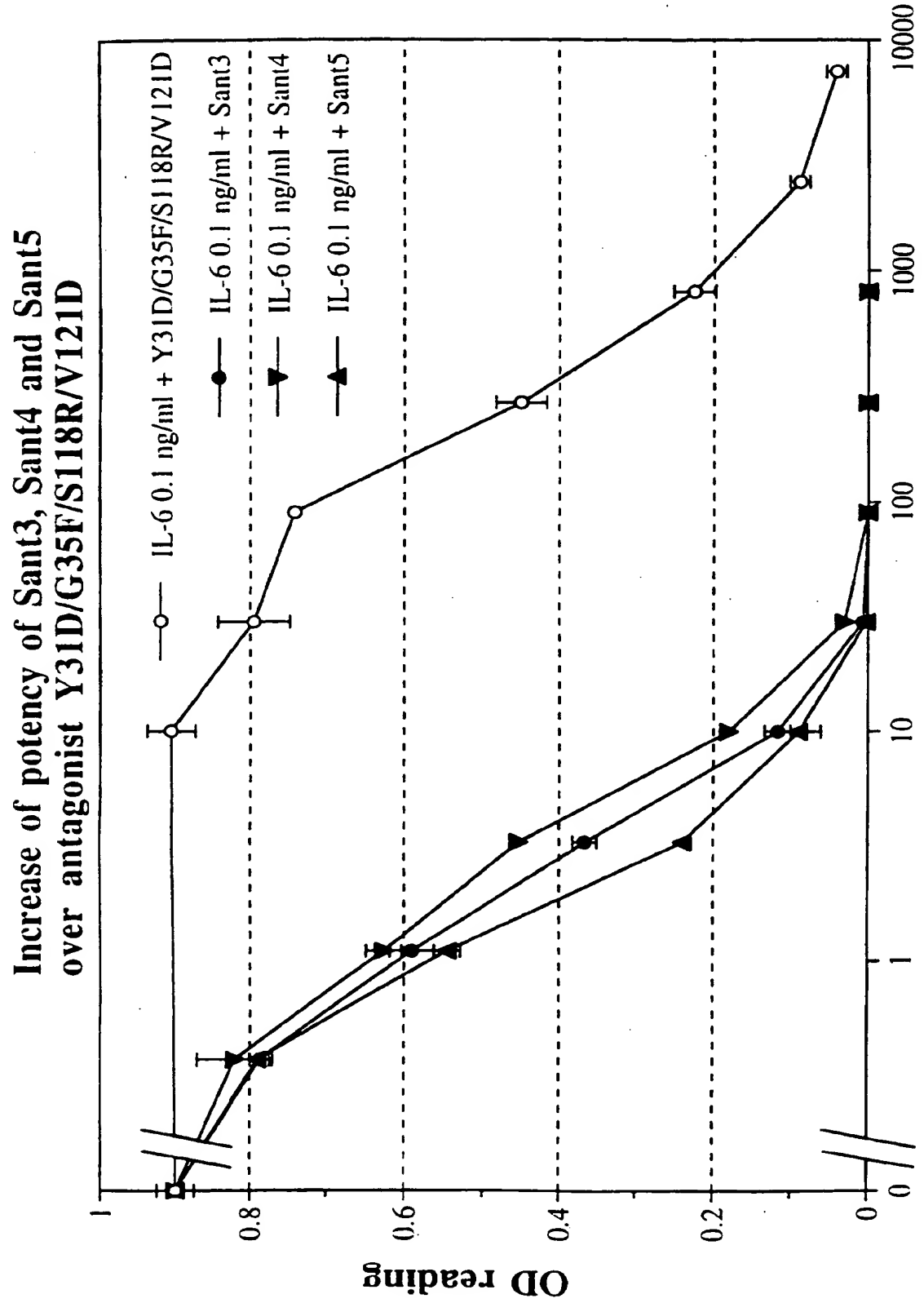
Increase of potency of Sant3, Sant4 and Sant5
over antagonist Y31D/G35F/S118R/V121D
IL-6 0.1 ng/ml + Y31D/G35F/S118R/V121D
IL-6 0.1 ng/ml + Sant3
IL-6 0.1 ng/ml + Sant4
IL-6 0.1 ng/ml + Sant5
OD reading
0
0.2
0.4
0.6
0.8
1
0
1
10
100
1000
10000
Concentration, ng/ml

FIG. 2

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/IT 95/00216

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/54 A61K38/20 C12N15/24 G01N33/74

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K C12N G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBO JOURNAL, vol. 13, no. 6, 15 March 1994 pages 1357-1367, XP 000565719 SAVINO, R. ET AL. 'Generation of interleukin-6 receptor antagonists by molecular-modeling guided mutagenesis of residues important for gp 130 activation' * whole disclosure * ---	1-6
X	PROC. NATL. ACAD. SCI. USA, vol. 90, 1993 XP 000565720 SAVINO, R. ET AL. 'Saturation mutagenesis of the human interleukin 6 receptor binding site: Implications for its three-dimensional structure' * abstract; figs. 1-3 * ---	1-3,5,6

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
26 March 1996	12.04.96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer Hermann, R

Form PCT ISA 210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int .onal Application No
PCT/IT 95/00216

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,94 09138 (CETUS ONCOLOGY CORPORATION) 28 April 1994 * claims 1-4 * ---	9,10,12
X	WO,A,94 11402 (ISTITUTO DI RICERCHE DI BIOLOGIA MOLECOLARE P. ANGELETTI S.P.A.) 26 May 1994 * page 1; claims * ---	1
A	J. IMMUNOL. , vol. 153, no. 4, 15 August 1994 pages 1744-1753, XP 000565715 EHLERS, M. ET AL. 'Identification of two novel regions of human IL-6 responsible for receptor binding and signal transduction' * abstract; p. 1746, left-hand column, last paragraph; p. 1749-1751 * -----	1-12

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int Jonal Application No
PCT/IT 95/00216

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9409138	28-04-94	AU-B-	5409294	09-05-94
		CA-A-	2147466	28-04-94
		EP-A-	0672144	20-09-95
WO-A-9411402	26-05-94	CA-A-	2129824	26-05-94
		EP-A-	0667872	23-08-95
		JP-T-	7501947	02-03-95